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Demonstration of reciprocal diurnal variation in human serum T3 and rT3 concentration demonstrated by mass spectrometric analysis and establishment of thyroid hormone reference intervals.

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Abstract

Background: There has been a wide range of reference intervals proposed in previous literature for thyroid hormones due to large between-assay variability of immunoassays, as well as lack of correction for collection time. We provided the diurnal reference intervals for five thyroid hormones, namely total thyroxine (TT4), total triiodothyronine (TT3), free thyroxine (FT4), free triiodothyronine (FT3), and reverse T3 (rT3), measured in serum samples of healthy participants using a liquid chromatography/tandem mass spectrometry (LC-MS/MS) method.

Methods: Couplet serum samples (a.m. and p.m.) were collected from 110 healthy females and 49 healthy males. Healthy volunteers were recruited from four participating centers between 2016 and 2018. Measurements of thyroid hormones were obtained by LC-MS/MS analysis.

Results: Our study revealed significant uptrend in AM to PM FT4 ($p < 0.0001$) samples, downtrend in AM to PM TT3 ($p = 0.0004$) and FT3 samples ($p < 0.0001$), and AM to PM uptrend in rT3 samples ($p < 0.0001$). No difference was observed for TT4 between AM and PM. No significant sex differences were seen for any of the five thyroid hormones.

Conclusion: When diagnosing thyroid disorders, it is important to have accurate measurement of thyroid hormones, and to acknowledge the diurnal fluctuation found, especially for FT3. Our study highlights the importance of standardization of collection times and implementation of LC-MS/MS in thyroid hormone measurement.

Keywords: diurnal variation, mass spectrometry, reference interval, thyroid hormone

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Introduction

Appropriate reference intervals are essential to clinicians when interpreting laboratory results on thyroid hormones.[1] However, current reference intervals for total thyroxine (TT4), total triiodothyronine (TT3), free thyroxine (FT4), and free triiodothyronine (FT3), when measured by immunoassays, are method-dependent because of the between-method variability.[1–3] Working groups from the International Federation of Clinical Chemistry (IFCC) have shown that the difference between immunoassays can affect establishing universal reference intervals that would apply across methods,[1] and, therefore, could negatively impact the clinical utility of immunoassays for thyroid hormones.

Measurement of thyroid hormones by isotope-dilution liquid chromatography/tandem mass spectrometry (ID-LC-MS/MS) offers superior

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specificity and accuracy compared with immunoassays, and therefore serves as the reference measurement procedure.[1,4–6] Whereas immunoassay measurements of free thyroid hormones are affected by thyroid hormone binding proteins such as thyroxine binding globulin (TBG), and, to a lesser degree, prealbumin and albumin, the interference is minimal when measured by LC-MS/MS after these proteins are removed by either ultrafiltration or equilibrium dialysis.[7–9] In addition, FT4 showed a far better inverse log-linear relationship with thyroid stimulating hormone (TSH) when measured by LC-MS/MS than by immunoassay,[7,8,10] suggesting inadequate measurement by immunoassays. This manuscript provides a comprehensive study to establish thyroid hormone reference intervals using LC-MS/MS.

TSH shows significant diurnal variation, and this could result in misclassification of patients if time-dependent (a.m. *versus* p.m.) reference intervals were not applied.[11,12] Similarly, diurnal variation was also seen in thyroid hormones, and especially in FT3.[13–15] Prior studies on reference intervals of thyroid hormones, however, have not corrected for collection time and this can potentially be one of the many reasons for the wide range in reference intervals.[1] This study provides the diurnal reference intervals for five thyroid hormones, namely TT4, TT3, FT4, FT3, and reverse T3 (rT3), measured in serum samples of healthy participants using the LC-MS/MS method.

Methods

Subjects and specimens

Subjects included 159 healthy volunteers; all were included after informed written consent was obtained. Healthy participants were determined by screening assessments including a detailed medical history, absence of medication or supplements use, and physical examination by a medical provider. We excluded participants with family history of thyroid disorders, smoking history, as well as measurable thyroid peroxidase (TPO) antibody values ($\geq$10.0 IU/ml on Siemens IMMULITE® 2000), which is associated with autoimmune hypothyroidism and Graves' disease.[16] Participants with a sample volume insufficient for TPO antibody measurement were also excluded. These volunteers lived in the United States, South Africa, and Turkey, where iodine intake is generally sufficient.[17–19]

Serum samples were collected at four sites, which included the National Institutes of Health (NIH, US), Georgetown University (US), Walter Sisulu University (South Africa), and Uludag University (Turkey) between 2016 and 2018. Morning samples were collected between 6:00 and 9:00 a.m., and paired evening samples were collected between 6:00 and 9:00 p.m. All blood sampling was carried out by professional phlebotomists, collected in preservative/gel-free serum specimen collection tubes, and kept chilled and centrifuged within 1 h of collection to separate serum. Samples were stored at −80°C until assayed by LC-MS/MS. All samples in this study were approved by the institutional review boards at NIH (protocol 93-CC-0094) and Georgetown University (Pro0000007-01).

LC-MS/MS

FT3 and FT4 were separated from protein-bound hormones by ultrafiltration at 37°C, followed by measurement using an ABSCIEX Triple-Quad-6500 isotope dilution LC-MS/MS System (AB Sciex, Concord, ON, CA) as previously described.[7,20] TT3, TT4, and rT3 were measured by atmospheric pressure photoionization LC-tandem MS using the Agilent 6460 triple-quadrupole mass spectrometer coupled to the Agilent 1200 Infinity Series HPLC (Agilent Technologies, Santa Clara, CA, USA) as previously described.[7,8] Complete method validation details have been published previously[20]; the intra-assay coefficients of variation are <9% for all five analytes. Inter-laboratory quality and accuracy of sample analysis was assessed prior to the study with the Mayo Clinic (Rochester, MN), NMS Laboratories (Willow Grove, PA) and Children's National Medical Center (Washington DC).

Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 8. Shapiro–Wilk normality test was used to assess distribution of thyroid hormone concentration as well as distribution of the data following log transformation. For Shapiro–Wilk test, alpha = 0.05 was chosen and the p value was compared with alpha to determine the passing of lognormality test. Difference

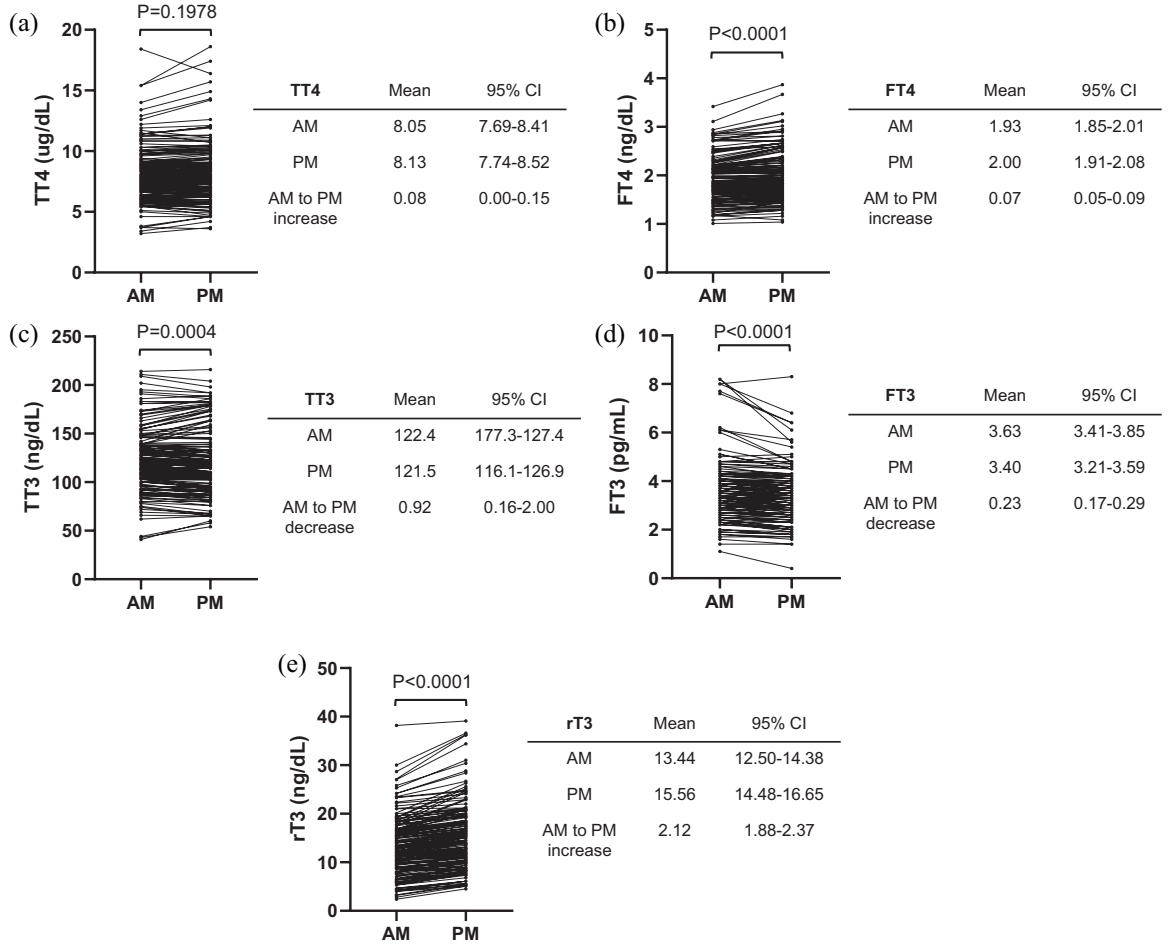

Figure 1. Paired results between a.m. and p.m. thyroid hormone values for (a) TT4, (b) FT4, (c) TT3, (d) FT3, and (e) rT3. *p* values were calculated on the basis of Wilcoxon tests.
FT3, free triiodothyronine; FT4, free thyroxine; rT3, reverse T3; TT3, total triiodothyronine; TT4, total thyroxine.

between a.m. and p.m. results within the same individual was assessed by Wilcoxon matched-pairs test. Reference intervals for all five thyroid hormones were obtained using the percentile approach. Significant values were determined at $p < 0.05$.

Results

The number of samples obtained for establishing the reference intervals for TT4, TT3, and rT3 was 159 pairs ($n = 110$ female; $n = 49$ male); due to sample volume shortage, the number was 146 pairs for FT4 ($n = 101$ female; $n = 45$ male) and 141 pairs for FT3 ($n = 99$ female; $n = 42$ male). Our study revealed significant uptrend in a.m. to p.m. FT4 samples ($p < 0.0001$), downtrend in a.m. to p.m. TT3 ($p = 0.0004$) and FT3 samples ($p < 0.0001$), and a.m. to p.m. uptrend in rT3 samples ($p < 0.0001$; Figure 1). No difference was observed for TT4 ($p = 0.1978$) between a.m. and p.m. Thyroid concentrations between the females and males were also compared. However, no significant sex differences were seen for any of the five thyroid hormones.

All five analytes (TT4, TT3, FT4, FT3, rT3) showed non-Gaussian distribution, and four analytes (TT4, TT3, FT3, and rT3) still exhibited non-Gaussian distribution following log transformation (Supplemental Table S1). Therefore, we recommend that the 2.5–97.5 percentile of the thyroid hormone values be used by laboratories routinely running this assay (Table 1). Due to the significant difference between a.m. and p.m. values, we recommended a.m. and p.m. reference intervals for FT4, TT3, FT3, and rT3 be reported based on time of sample collection (Table 1).

Table 1. Recommended reference intervals for thyroid hormones.

Percentile	**2.5th**		**97.5th**		***n***
TT4 (ug/dl)	4.6		12.9		318
	a.m.		**p.m.**		
Percentile	**2.5th**	**97.5th**	**2.5th**	**97.5th**	***n***
FT4 (ng/dl)	1.18	2.88	1.22	3.13	146
TT3 (ng/dl)	62	202	65	192	159
FT3 (pg/ml)	1.8	7.6	1.7	6.1	141
rT3 (ng/dl)	4.0	195	5.4	192	159

FT3, free triiodothyronine; FT4, free thyroxine; rT3, reverse T3; TT3, total triiodothyronine; TT4, total thyroxine.

Discussion

Measurement of thyroid hormones in conjunction with TSH is used for the diagnosis and management of thyroid disorders. These analytes are routinely measured using automated immunoassay because the method is generally precise, quick, and does not require extensive training of personnel. However, immunoassays exhibit many limitations and often do not correlate with ultrafiltration LC-MS/MS or the patient's clinical presentation. For example, the one-step direct analog immunoassays are used most frequently to measure free thyroid hormones, but they are affected by changes in serum binding proteins.[21] Severe binding protein abnormalities, such as congenital TBG excess or deficiency,[22,23] dysalbuminemias,[24,25] high TBGs in pregnancy, and low TBGs in patients with renal diseases and proteinuria, as well as thyroid hormone autoantibodies,[26,27] can all cause interference in FT4 and FT3 immunoassays. Immunoassays are also impacted by non-thyroid hormone related interferences from heterophile antibodies, including human anti-mouse antibodies (HAMA) and rheumatoid factor (RF), as well as interference related to the use of supplements such as biotin.[1] All of these issues make harmonization a goal difficult to achieve.[9]

To overcome these limitations, we have developed two thyroid profile assays, one for the analysis of TT4, TT3, and rT3 utilizing LC-MS/MS, and the other for the simultaneous analysis of FT4 and FT3 using ultrafiltration followed by LC-MS/MS.[28] Compared with immunoassay, thyroid hormone results measured by LC-MS/MS correlate better with patients' clinical conditions, and especially in patients with hypothyroidism and hyperthyroidism.[21,29,30] Thus, to date, this method is the preferred way to measure thyroid hormones accurately and precisely. However, accurate diagnosis should also be accompanied by establishment of reference intervals that adequately control for diurnal fluctuation of thyroid hormones.[31]

According to our observations, one of the biggest diurnal changes was seen in FT3, and the amplitude and timing of variability are consistent with published studies.[13] With T3 being the biologically active hormone, assessment of its concentration while applying the appropriate reference range could provide valuable information, especially under pathophysiological conditions where serum FT3 levels are outside of the reference range whereas T4/TSH levels are still normal.[15] Some of these conditions include nonthyroidal illness, targeted inactivation of TRH gene, and caloric restriction.[15,32]

Our results suggest that FT4 has a significant uptrend at the end of the light phase (early evening) compared with the beginning of the light phase (early morning), which follows the rhythmic release of TSH as we have previously shown in this population.[12] In addition, the higher concentration of steroids in the morning can inhibit TSH, whereas steroids reach the lowest levels in the evening, which results in an increase in TSH.[30] At the same time, an opposite downward trend was observed for TT3 and FT3. It was previously shown that type II 5'-iodothyronine

deiodinase (5'D-II), the enzyme that catalyzes the intracellular conversion of T4 to T3 in the central nervous system, exhibited a significant daily variation, with a maximum in the early morning, and a minimum in the early afternoon.[33] This change in 5'D-II activity could account for the opposite trends we observed in FT4 and FT3. On the other hand, type III 5-iodothyronine deiodinase (5D-III), the enzyme responsible for converting T4 to rT3, did not show diurnal variation, which is consistent with the similar upward trend in rT3 and FT4.[33]

It should be noted that, in our study, FT3 and FT4 were separated from protein-bound hormones by ultrafiltration at 37°C. Since ultrafiltration is temperature sensitive,[34] for ultrafiltration performed at ambient temperature (25°C), the reference interval should be adjusted accordingly. It has been shown that results from LC-MS/MS following ultrafiltration at 37°C correlate well with those prepared by equilibrium dialysis,[23] so our proposed reference intervals would potentially apply to separation by equilibrium dialysis.

Although the importance of reference intervals has been highlighted here, our results might also be significant in advancing individualized care. For patients undergoing thyroid hormone replacement therapy, for example, the detection of subtle changes in thyroid hormones is made possible through such technologies as LC-MS/MS.[13,35] At the same time, this new set of time-specific reference intervals could better reflect the normal circadian rhythm of thyroid hormones, and its application would allow optimized medical care.[13] Studies have also shown that many patients diagnosed with subclinical hypothyroidism based on FT4 and FT3 immunoassay results had free thyroid hormone values below the reference interval when measured by LC-MS/MS.[9,21,29] In this group of patients, for example, accurate measurement of free thyroid hormones by LC-MS/MS and application of the appropriate reference range permit more effective clinical decision making and may shift medical intervention from the watch-and-wait strategy to more prompt treatment.[9]

In our study, we observed large variations in thyroid hormone concentrations among individuals (Figure 1), similar to those seen in previous reference interval studies.[1,36] However, it could be argued that this high individuality can cause our proposed reference ranges to be insensitive to changes in test results that are significant for the individual. To overcome this large variation, a previous study has shown that subject-based reference intervals can be more informative than population-based reference intervals, and especially in diagnosing patients with subclinical thyroid disease.[36] Our findings on average a.m. to p.m. change can be important when a subject-based reference interval is implemented since diurnal difference is a major component of within-individual biological variation.

In summary, this study establishes the time-specific reference intervals for LC-MS/MS-based measurements of five thyroid hormones. These reference intervals were implemented at the Clinical Center, NIH in June, 2019, and we strongly recommend other institutions consider using the far more specific LC-MS/MS approach than the regularly employed immunoassay methods, as the latter lack specificity and frequently could provide wrong clinical classification.

Author contribution(s)

Qian Sun: Conceptualization; Data curation; Formal analysis; Resources; Writing-original draft; Writing-review & editing.

Lívia Avallone: Conceptualization; Data curation; Resources; Writing-review and editing.

Brian Stolze: Data curation; Formal analysis; Methodology; Writing-review and editing.

Katherine A. Araque: Data curation; Investigation; Resources; Writing-review and editing.

Yesim Özarda: Data curation; Methodology; Resources; Writing-review and editing.

Jacqueline Jonklaas: Data curation; Investigation; Resources; Writing-review and editing.

Toral Parikh: Data curation; Methodology; Resources; Writing-review and editing.

Kerry Welsh: Data curation; Investigation; Resources; Writing-review and editing.

Likhona Masika: Data curation; Methodology; Resources; Writing-review and editing.

Steven J Soldin: Conceptualization; Data curation; Project administration; Resources; Writing-original draft; Writing-review and editing.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Supplemental material

Supplemental material for this article is available online.

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